BRET analysis of GPCR oligomerization: newer does not mean better.

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« Rigorous » BRET analysis of GPCR oligomerization: Newer does not mean better.

James et al.(1) proposed a "rigorous" treatment of bioluminescence resonance energy transfer (BRET) data to distinguish random (non-specific) from true oligomeric protein interactions. The question is not trivial and the intention laudable since BRET has become increasingly popular with more than 100 original articles published. However, the authors dismissed many studies that addressed similar issues and several points deserve comments.

The authors preface their study by the statement: "Conventional BRET experiments are presently done at high levels of expression and at a single acceptor:donor ratio". Although it is true of some studies, such general statement ignores a large body of work where these parameters were taken into account. Expression levels were monitored in many studies and found to be within physiological range (2-5) and at least 15 papers performed BRET titration assay where the ratio donor:acceptor was varied (ex: 2,4-6). Specificity of the BRET signals allowing to distinguish oligomerization from random collisions has also been verified by several authors using BRET competition assays where the occurrence of BRET between two partners expressed at a given donor:acceptor ratio can be inhibited by expression of the non-tagged partners but not of a non-tagged non-interacting protein (ex: 3,6-8).

James et al. propose to differentiate random from true oligomeric interactions based on theoretical considerations summarized in a seminal article by Kenworthy and Edidin (9). The first approach consists in studying BRET efficiency ($BRET_{eff}$) in experiments where the acceptor:donor (GFP/Luc) ratio is varied (type I assay). Random interactions are expected to be less sensitive to the acceptor:donor ratio if the surface density of the acceptor remains low. In multiple previous studies reported in the literature, the change of acceptor:donor ratio was obtained by maintaining the donor concentration fixed and progressively increasing the acceptor and true oligomeric interactions deduced from the hyperbolic progression of the BRET (ex: 2,4-6). In the experiments conducted by James et al., the total concentration of acceptor+donor was maintained constant by inversely changing the concentrations of both donor and acceptor and the difference between pseudo- and true- hyperbolic BRET curves used to define random collision. Such analysis is technically difficult and complicated by the fact that the efficiency of transfer for random collisions becomes independent of donor/acceptor ratio only if the acceptor concentration is kept constant (see table I in ref 9).

In type I assays, James et al. also interpreted lower maximal BRET value as evidence for the lack of dimerization or equilibrium between dimers and monomers. Such interpretation is very hazardous given that the extent of RET signals vary with the distance between donors and acceptors within a dimer. Thus no direct conclusion can be drawn on the amount of dimers simply based on the maximal BRET signals observed.

In type II assay, BRET for true oligomers should be independent of the concentrations of BRET partners at a fixed acceptor:donor ratio. This was previously shown to be the case for class A GPCRs (1, 11, 12) and in particular for the ß2AR at receptor concentration below 15pmol/mg of protein (1). From their data, James et al. concluded otherwise. However, a close examination of Fig 4, reveals that the ß2AR BRET curve has a slope that appears closer to that of the constitutive CTLA-4 dimer than that of the CD2 or CD86 monomers, consistent with the notion that the BRET between ß2AR-luc and ß2AR-GFP may reflect constitutive oligomerization. Also, the BRET signal observed for the constitutive dimer CD80 increase more readily with increasing expression levels than that of the ß2AR, further complicating data interpretation. The fact that BRET falls below detection level at low donor/acceptor ratio may reflect lack of detector sensitivity for pairs yielding low BRET signals. Despite these interpretational difficulties, the authors concluded that the entire concept of GPCR oligomerization needs reappraisal.

In their discussion concerning the specificity of the BRET signals observed in previous studies, James et al. argued that the GABAb type-2 receptor (GBR2) is a poor choice because it can itself dimerize. The reason to use this receptor as a negative control was precisely its demonstrated ability to dimerize thus offering a

reliable selectivity test using a dimerization competent receptor. Also, contrary to what was implied by the authors, GBR2 is not the only negative control that was used in BRET studies. A number of other receptors were used as negative controls in BRET studies addressing class A GPCR oligomerization (ex: 3, 13-15).

The observation that in some studies, ligand binding affects the maximal BRET signal between the proposed protomers of class A GPCR oligomers, is difficult to reconcile with the implicit conclusion of James et al. that the BRET signals observed for class A GPCRs most likely result from random collisions. In many of these studies, the ligand-promoted changes in BRET signal were interpreted as conformational changes within pre-existing dimers that changed the distances between the energy acceptor and donor.

Finally, the notion that family A GPCRs may form constitutive oligomers is not only based on BRET studies. Many other biochemical and biophysical approaches support this notion. These include: co-immunoprecipitation, various types of FRET, atomic force microscopy, covalent cross-linking, gel filtration, neutron scattering experiments, functional complementation, cell biology studies demonstrating cross-internalization and co-processing of GPCRs as well as binding studies showing positive and negative cooperativity. These approaches, their relative strengths and caveats, including methodological considerations and potential functional outcomes have recently been reviewed (16, 17). It is therefore premature to dismiss the GPCR oligomer hypothesis based on the interpretations of a single BRET study.

In conclusion, we believe that the results reported in the article by James et al. can be interpreted in different ways and that more controls would have been necessary to challenge the multidisciplinary work conducted on this topic by many groups over the past 10 years. Clearly, BRET is gaining in popularity to assess protein-protein interaction in living cells and additional quantitative approaches will certainly be forthcoming. Maybe more importantly, additional studies performed in native tissues are needed to establish the generality of GPCR dimerization in physiologically relevant systems.

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